UNIVERSITI PUTRA MALAYSIA

QUANTIFICATION AND CHARACTERIZATION OF TRICHODERMA SPP. FROM DIFFERENT HABITATS

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QUANTIFICATION AND CHARACTERIZATION OF *TRICHODERMA* SPP. FROM DIFFERENT HABITATS

By

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THE QUANTIFICATION AND CHARACTERIZATION OF *TRICHODERMA* SPP. FORM DIFFERENT HABITATS

By

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November 2002

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The abundance of *Trichoderma* was not significantly different between the oil palm cultivated areas and jungle areas. Soil pH and soil moisture content did not have an effect on the abundance of *Trichoderma* in the areas sampled. *Ganoderma* infected area with percentage disease incidence (PDI) of > 30% recorded higher frerquency (9.5 x 10^3 cfu/g air dried soil) of isolation of *Trichoderma*. In the reserved forest habitat, inland soil seemed to harbor higher population (10.9 x 10^3 cfu / g dried soil) of *Trichoderma*. Generally for all habitats and areas sampled, the two upper soil horizons (Al and Be) supported higher population of *Trichoderma* and the distribution decreased with depth of soil. However, in the EFB mulched area there was a significant increase in *Trichoderma* with increase in depth of profile. Based on phenotype appearances, four species aggregates of *Trichoderma* were identified from oil palm and forest rhizospheres, namely *T. harzianum*, *T. virens*, *T. koningii*, and

T. longibrachiatum. *T. harzianum* and *T. virens* were the most frequently isolated species aggregates while *T. longibrachiatum* was the least. The variation between species aggregates of *Trichoderma* was distinguished by using RAPD. However, overlapping was found between *T. virens* and *T. koningii* and *T. longibrachiatum* within a main cluster. Isolates of the same species were group together within the same sub cluster indicating a close genetic linkage among the same species. Several putative DNA markers were identified that could be used for interspecies differentiation if consecutive PCR tests were carried out with primer OPC-11 and OPC-15. Confrontation assay based on percentage inhibition of mycelial growth and colony overgrowth showed that there were variations in the degree of antagonistic ability between and within species aggregates of *Trichoderma*. The mode of action was attributed to competition, mycoparasitism and / or antibiosis. Isolates TH80 of *T. harzianum*, TK126 of *T. koningii* and TV26 of *T. virens* were found to be the most effective antagonists.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan ijazah Master sains

KUANTIFIKASI DAN PENCIRIAN *TRICHODERMA* SPP. DARI HABITAT YANG BERBEZA

Oleh

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November 2002

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Perbezaan bilangan *Trichoderma* di antara kawasan kelapa sawit and hutan adalah tidak bermakna. pH tanah and kelembapan tanah daripada kawasan persampelan tidak memberi kesan kepada bilangan *Trichoderma.* Kawasan yang dijangkiti *Ganoderma* dengan peratus kejadian penyakit (PDI) > 30% mencatatkan kekerapan pengasingan *Trichoderma* yang tinggi Sementara itu di dalam hutan simpanan, tanah kawasan pedalaman memendam populasi *Trichoderma* yang lebih tinggi (10.9 x 10^3 cfu / g tanah udara-kering) . Umumnya, bagi semua habitat dan kawasan persampelan, tanah di bahagian atasan menyokong populasi *Trichoderma* yang lebih tinggi dan taburannya menurun selari dengan kedalaman profil tanah. Walaubagaimanapun, di kawasan sungkupan EFB terdapat penambahan bermakna dalam bilangan *Trichoderma* selari dengan kedalaman tanah. Berdasarkan kepada ciri-ciri phenotip, 4 jenis spesies agregat telah dikenalpasti dari rizosfera kelapa sawit and hutan, yakni *T. harzianum*, *T. virens*, *T. koningii* dan *T.longibrachiatum. T. harzianum* merupakan spesies agregat yang paling banyak diasingkan manakala

T. longibrachiatum paling sedikit. Perbezaan di antara spesies agregat *Trichoderma* dapat dibezakan dengan RAPD. Walaubagaimanapun, pertindihan didapati berlaku di antara *T. virens* dengan *T. koningii* dan *T. longibrachiatum* di dalam rumpun utama. Namun demikian, isolat dari spesies yang sama dikumpulkan di dalam sub-rumpun yang sama menunjukkan hubungan genetic yang erat di kalangan isolat yang sama spesies. Beberapa penunjuk anggapan DNA telah dikenalpasti untuk pembezaan interspesies jika ujian PCR berterusan dijalankan dengan prima OPC-15 dan OPC-15. Ujian konfrontasi berdasarkan kepada peratusan perencatan pertumbuhan miselium dan pertumbuhan koloni menunjukkan bahawa terdapat tahap keantagonisan yang berbeza di antara dan di kalangan spesies agregat *Trichoderma*. Cara tindakan dikaitkan dengan persaingan, mikoparasitisme, dan / atau antibiosis. Isolat TH80 dari *T. harzianum*, TK126 dari *T. koningii* dan TV26 dari *T. virens*, merupakan antagonis yang paling berkesan.

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I certify that an Examination Committee met on 11th April 2003 to conduct the final examination of Choo Chee Wei on his Master of Science thesis entitled "Quantification and Molecular Characterization of *Trichoderma* spp. from Different Habitats" in accordance with the Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

(CHOO CHEE WEI)

Date: 18 JUL 2003

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LIST OF ABBREVIATIONS

µl	micro litre
^0C	degree celcius
%	Percentage
ANOVA	Analysis Of Variance
bp	base pair
cfu	Colony forming unit
cm^2	centimeter square
dATP	Deoxyadenosine Triphosphate
dCTP	Deoxycytidin Triphosphate
dGTP	Deoxyguanisine Triphosphate
dTTP	Deoxythymidine Triphosphate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
g	gram
GSM	*Ganoderma* Selective Medium
K_2HPO_4	Dipotassium hydrogen phosphate
KCL	Potassium chloride
LCB	Lactophenol blue
MEA	Malt extract agar
$MgSO_4.7H_2O$	Magnesium sulphate
mL	mililitres

NH_4NO_3	Ammonium nitrate
PCNB	Pentachloro-nitrobenzene
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PIRG	Percentage Inhibition of Radial Growth
RAPD	Random Amplified Polymorphic DNA
SDS	Sodium Deodecyl Sulfate
Taq	Thermal aquatius
TH	*T. harzianum*
TK	*T. koningii*
TL	*T. longibrachiatum*
TME	*Trichoderma* Medium E
TV	*T. virens*
v/v	volume per volume

CHAPTER 1

INTRODUCTION

African oil palm, *Elaeis guineensis* Jacq., is one of the most important plantation crops in Malaysia. It produces palm oil and palm kernel oil, which are widely used in food and other industries such as detergents and cosmetics. Malaysia is the world's largest producer and exporter of the oil, accounting for more that 50% of the world's oil and fat production. The total area of oil palm plantations is close to 3.4 million hectares, which account for almost 50% of the land under cultivation in Malaysia (Malaysia Palm Oil Statistic, 2001)

The oil palm industry in Malaysia is being threatened by Basal Stem Rot (BSR), a disease commonly associated with areas where oil palms have been planted after coconut, especially on clay soils in coastal areas. It is caused by species of *Ganoderma*. It was concluded that old oil palms over 30 years old were those most commonly affected by BSR although reports 5 years old palm and younger and has been detected to be prone to *Ganoderma* infection even in peat and inland soils. Normally the disease progress is slow but this is not always the case, especially in the second-generation palms, the progress increase by 50%. Control measures such as clean clearing, surgery and fungicide were found to be unsuccessful against *Ganoderma*. The success of biological control for numerous pathosystem has shifted the interest to explore

the potential of BSR control through manipulation of antagonistic microorganism such as species of *Trichoderma*.

The pioneering work of Rifai (1969) in distinguishing nine species aggregates has been the basis for identification in *Trichoderma*. *T. virens, T. harzianum, and T. viride* have been reported as the most common biological control agents of the genus *Trichoderma*.

There is limited understanding of the population dynamics of *Trichoderma*, its survivability and proliferation in relation to soil type, soil depth and cropping history in the local ecosystem. Since *Trichoderma* are applied outside the plant, and mode of action by competition, mycoparasitism and possibly antibiosis, its ability to disperse and to colonize roots will determine its effectiveness as biocontrol agents. Thus, the understanding on the quantitative and qualitative distribution of *Trichoderma* in different ecological niches is essential before they can be developed into biological formulation for field application. Different strains within the same species aggregates showed different degree of adaptation to different soil types, environmental conditions and rhizosphere competency. This is the reason why the disease controlling ability of *Trichoderma* varied from place to place.

Classification of *Trichoderma* species, and the ability to distinguish one strain from another, are very important issues in the field of biological control. Identification of *Trichoderma* aggregates base on morphological descriptions of

colony growth and conidiospores is highly artificial. In recent years there has been vast progress in the development of molecular biological tools and technologies. These have been increasingly applied to the study of fungal plant pathogens. The development of techniques in molecular biology have provided many new tools for the identification of specific strains among strains of same species. These include Random Amplify Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Microsatelite and ribosomal DNA (rDNA) sequences analysis. RAPD and rDNA sequences analyses are the methods that have been proven to enable such identification.

Therefore, the objectives of the present study are:

- To quantify the population dynamics of *Trichoderma* spp. from different oil palm and forest ecosystems
- To characterize the variation between and within species of *Trichoderma*
- To evaluate the antagonistic activity of representative isolates of *Trichoderma* against *Ganoderma in-vitro*

CHAPTER 2

LITERATURE REVIEW

Basal Stem Rot of Oil Palm

The Basal Stem Rot (BSR) disease has long been recognized to be the most annihilating disease of field palms in South East Asia. It still reigns as the number one killer of oil palms (*Elaeis guineensis* Jacq.) and a disasterous blow to Malaysia's palm oil production (Azizah, 2002). Besides Malaysia, BSR of oil palm has also been reported in Zimbabwe and Tanzania in Africa (Turner, 1981), Honduras in Central America (Chincilla and Richardson, 1987), Thailand (Tummakate and Likhitekaraj, 1994), Colombia (Nieto, 1995), and Singapore (Ariffin and Idris, 2002).

The BSR disease was first described in the Republic of Congo, West Africa in the year 1915 (Wakefield, 1920). Thompson (1931) was the first person to record the incidence of basal stem infection of oil palms by *Ganoderma* in Malaysia. Several *Ganoderma* species particularly *G. lucidum* (Navaratnam, 1961,1964; Turner, 1965) have been reported to be the causal agent of the disease. However, reports of Steyaert (1967, 1972) showed that *G. lucidum* is confined mainly to temperate regions. Hence, it was suspected that *G. lucidum* was not the exact species pathogenic to oil palm.

Since 1931, basal stem rot continues to be the most serious disease of oil palm in Malaysia, causing significant yield losses. Ho and Nawawi (1985) concluded from their study that *G. boninense* was the causal pathogen associated with basal stem rot of oil palm in Peninsular Malaysia. However, previous reports by other researchers (Varghese *et al.*, 1975; Turner, 1981, Ariffin, 1989a, 1989b) suggested that several species may be involved in causing the disease but whether the species are all equally virulent and whether dual or multiple infection can occur are not known (Turner, 1981). More recently, Idris *et al.* (2000a) identified four species of *Ganoderma* (*G. boninense, G. zonatum, G. miniatocintum and G. tornatum*) to be associated with BSR of oil palm in Peninsular Malaysia, with the latter found to be non-pathogenic (Idris et al., 2000b). However, the study conducted by several independent researchers (Khairuddin, 1991; Sariah *et al.*, 1994; Ariffin *et al.*, 1995 and Teh 1996) with the adoption of reliable pathogenecity inoculation technique and isozyme characterization (Faridah, 1994) concluded that *G. boninense* was the species that is specifically pathogenic to oil palm.

Before 1957, BSR incidence was thought to be economically unimportant as only very old palms of over 25 years were infected (Turner, 1981). The fructification of the fungus was recognized and accepted as normal development resulting from increasing age and senescence of the palms (Turner, 1965). Towards the later years in 1960s, when oil palm began to assume prominence as a plantation crop, BSR incidence was on the increase infecting much younger palms of 10 to 15 years old (Turner, 1981). It was later

reported that the disease could set in as early as 12 to 24 months but are more frequent on 4 to 5 years old palms, particularly in replanted areas (Singh, 1991) or under-planting with coconut palms (Ariffin *et al.*, 1996). In replanting from jungle and rubber, BSR begins to develop when the palms are about 10 to 12 years old (Singh, 1991). The BSR incidence is low initially (1 - 2%), but increase to 25% by the time the palms reached 25 years and are ready for replanting. In replanting from coconut and oil palm, the disease incidence was more than 50% after the 15th year.

High incidence of BSR disease was recorded on oil palms in coastal soil in west Penisular Malaysia (Ariffin and Idris, 2002). Although peat soils were once thought to be nonconducive to BSR incidence (Turner, 1981), serious incidences of the disease have been reported (Ariffin *et al.*, 1989a; Rao, 1990) in these soil. Ariffin *et al.* (1989a) concluded that *Ganoderma* poses a threat to oil palm plantings in peat soils where high incidence of the disease have been observed at a relatively young age, irrespective of previous cropping history. BSR disease was also recorded in inland soils (Khairudin, 1990) but the incidence was relatively low and seems to be confined only to waterlogged areas. However, the disease was recently reported on oil palms growing in lateritic soils, which was previously almost disease free (Benjamin, 1993; Benjamin and Chee, 1995).

It has long been accepted that natural infection with *Ganoderma* starts when the roots of oil palm coming into contact with BSR-affected debris within